ORIGINAL ARTICLE

DIAGNOSIS OF GASTROINTESTINAL TUBERCULOSIS: USING CYTOMORPHOLOGICAL, MICROBIOLOGICAL, IMMUNOLOGICAL AND MOLECULAR TECHNIQUES - A STUDY FROM CENTRAL INDIA

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ABSTRACT

The present study included three groups: (A) age and gender matched control (n=24) with no previous signs of *M. tuberculosis* complex (MTBC) infection, (B) patients (n=28) diagnosed with gastro-intestinal TB (GITB), (C) patients (n=50) with clinical and histo-pathological signs of GITB, but were culture and AFB negative. Real time assay performed using fluorescence resonance energy transfer hybridization probes showed a positivity index of 36 % in group C, i.e. 18 were found reactive from the total 50 cases studied. In addition, immune characterization of these 18 cases showed depleted CD₄⁺ count and increased levels of IFN- γ and TNF- α cytokines. No positive case was found in group A, while in group B, out of total 28 cases studied 27 were found positive. A combinatorial diagnostic approach for rapid detection and characterization of GITB might provide specific therapeutic strategies for prevention and treatment of the infection in future.

KEY WORDS

Mycobacterium tuberculosis, Immuno-phenotyping, Intestinal tuberculosis, Real time PCR, Cytokines.

INTRODUCTION

Mycobacterium tuberculosis complex (MTBC) is a group of ubiquitous intracellular pathogens responsible for 2-3 million deaths per year. India reported a significant high morbidity and mortality due to tuberculosis (TB) contributing one-fifth of the global cases with 325 172 deaths reported in the 2005 cohort (1, 2). In spite of its high risk of morbidity and mortality, depending on the immune status of the patient, it can also manifest in extra pulmonary sites of the body such as gastrointestinal (GI) tract, lymph nodes or solid viscera. Although, studies have suggested that the sites of extra-pulmonary tuberculosis (EPTB) may vary, GI tract remains to

be a major extra pulmonary site as its incidences has been found to be increasing through the decade (3).

The diagnosis of gastrointestinal tuberculosis (GITB) remains to be a major challenge. A typical clinical presentation of intestinal TB includes abdominal pain, weight loss, fever, weakness, nausea, vomiting, obstruction and bleeding. Intestinal TB often mimics inflammatory bowel disease or malignant neoplasia and its preoperative diagnosis is a challenge (4). Immunological structures (granulomas) which form in the infected site in response to persistent antigen and cytokine signals may be used for diagnosis but might overlap with that of other infectious and non infectious diseases (5, 6). Moreover, traditional bacteriologic studies including the microscopic demonstration of acid fast bacilli (AFB) and MTB culture are time consuming and lack sensitivity (7) thereby reinstating the need for more faster and effective diagnostic tools. Diagnosis of MTB specific DNA sequences represents a rapid and sensitive method (8). Conventional PCR methodology like single step PCR, nested PCR, though provide a likely strategy, but pose an enhanced risk of sample

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contamination instead (9). In this aspect, real-time PCR technology serves as an apt alternative, since PCR and fragment analysis are performed in a single closed tube thereby minimizing the risk of contamination.

Furthermore, clinical manifestations of MTB infections are variable and depend on a number of factors that are related to the microbe, the host and the environment (10). The host immune responses influence the disease manifestation and difference in the retort of immune components is likely to determine the disease progresses, resolves, or becomes latent (11). Complete eradication of MTBC is a result of a successful immune response that requires priming and activation of antigen-specific CD_4^+ and CD_8^+ T lymphocytes, their recruitment to the site of infection and production of cytokines leading to inhibition or killing of bacilli (12, 13). Although, this response helps to limit bacterial replication and dissemination *in vivo*, it also causes significant immuno-pathology and is suppressed (14). Despite a clear increase in the frequency of EPTB in immuno-suppressed patients, the clinical features of intestinal tuberculosis are seen rarely (4).

In order to expand the knowledge base regarding to diagnosis of GITB the present investigation was performed. The diagnosis of the culture negative GITB infections was performed by using Real Time PCR (Light Cycler 2.0) from formalin-fixed, paraffin-embedded tissues and endoscopic biopsies and later the characterization of the host immune status through analysis of Th1/Th2 cytokines and immuno-phenotyping were done in the PCR positive samples.

MATERIALS AND METHODS

Subject selection: The study was approved by the Institutional Review Board (IRB), Bhopal Memorial Hospital and Research Centre, Bhopal (India). Informed consent was obtained from human subjects included in the study and all clinical information pertaining to it was properly recorded. There was no serologic evidence of co-infection with other hepatotropic viruses.

Reagents: For isolation of MTBC DNA DNeasy Blood & Tissue kit (QIAGEN, Hilden, Germany) was used. Real-time assay was performed in thus extracted DNA with the help of LC PCR MTB detection kit using Roche Light Cycler 2.0 (LC) (Roche Molecular Diagnostics, Mannheim Germany). Lymphocyte enumeration studies were performed following whole blood lyses protocol using Tri test Trucount tubes from BD™ Biosciences (San Diego, CA, USA). For the analysis of Th1/Th2 cytokines, the BD™ Multiplex Cytometric Bead Array

(CBA) kit from BD™ Biosciences (San Diego, CA, USA) was used.

Specimen collection: A total of 102 samples were included in the study which was divided into three groups- (A) age and gender matched controls (n=24) with no previous signs of MTBC infection (B) The patients in group B (n=28) were known positives for the intestinal TB based on gold standard microbiological AFB (Ziehl Neelson) stain and culture methods (C) This group consisted of culture and AFB negative patients (n=50) suspected to be GITB positive as they showed clinical and histo pathological signs of TB with no co-existence of pulmonary and HIV infections. The inclusion criteria of the patients in group C was chosen according to the presence of certain symptoms, namely, abdominal pain, dyspepsia, weight loss, fever, anorexia, nausea and vomiting, constipation, chronic or bloody diarrhea, change in bowel habits, mal-absorption, and additional suspicious lesions in other body parts. The formalin-fixed, paraffin-embedded tissue and endoscopic biopsy samples were obtained from Medical & Surgical Gastroenterology Departments of Bhopal Memorial Hospital & Research Centre and stored at -20°C (processed within 7 days). 10 ml of EDTA mixed blood was collected from LC PCR GITB positive patients of group C and age and gender matched healthy volunteers by routine venipuncture method for immunophenotyping and analysis of Th1/Th2 cytokines. The control blood was considered healthy following routine laboratory analysis.

Microbiological examination: The BacT/ALERT 3D system (bioMérieux Inc., North Carolina, USA) was used for the culture based detection of MTBC. The method is based on the detection of carbon dioxide (CO_2) released by actively proliferating *Mycobacteria*. The elevated CO_2 concentration lowers the pH in the medium, which in turn produces a color change in a sensor in the vial, which is detected by a reflectometric unit in the instrument. The BacT/ALERT automatically performs readings every 10 min. and all data are transferred to and saved in the BacT/VIEW data management system. The classic Ziehl-Neelson technique was used for the acid-fast staining; the smear was flooded with carbol-fuchsin dye, heated and then decolorized. The non-acid fast organisms were then counterstained with methylene blue. Acid-fast MTBC appear red due to the retention of the carbol-fuchsin while the background and any non-acid-fast organisms will appear blue due to the methylene blue counter stain.

Quantification of MTB DNA through LC PCR: Formalin-fixed and paraffin-embedded (FFPE) specimens were sliced

with disposable sterile blades in each paraffin block, deparaffinized twice with 1 ml xylene and ethanol (100%), whereas for the formalin-fixed tissue biopsy and endoscopy samples, the specimens were washed twice in PBS prior to DNA isolation and DNA was extracted from the samples by Proteinase K digestion in combination with DNeasy Blood & Tissue kit following the tissue protocol. Real-time assay was performed in thus extracted DNA with the help of FRET probes using LC PCR 2.0 following all necessary instructions. A universal set of primers directed towards most conserved region of the 16S rRNA gene of the MTBC was used. The sequences of the primers, which recognized a 206-bp region of the 16S rRNA gene, were 5'-ACGGAAAGGTCTCTTCG-3' and 5'-CTTGGTAGGCCGTCAC-3'. The sequence of internal control (IC) oligonucleotide used was 5'-ACGGAAAGGTCTCTTCGAGATACTCGAGTGGCGAACGGGTGAGTAACA CGTGGGTGGGAAGCATGTTTTGTGGTGTAAGCGCTTAG CGGTGTGGGATGAGCGTGACGGCCTACCAAG-3'. Working master mix was prepared as per the protocol by adding 13.5 µl master mix concentrate, 2 µl Mg²⁺ and 0.5 µl IC. Finally 15 µl of working master mix and 5 µl of the sample were dispensed in each capillary and were run on LC PCR. Absolute quantification of bacterial load was performed by using Light cycler software 4.0 with appropriate quantitative standards (MTBC positive controls) and by following the guidelines for the quantitative analysis on the Light Cycler 2.0 instrument. Quantitative bacterial load means the total amount of bacterial DNA present in the given sample expressed in copies/µl.

Lymphocyte subset enumeration studies

(Immunophenotyping): The group C samples (culture/AFB negative) reported positive by LC PCR were further subjected for immunophenotyping. Estimation of lymphocyte subset population was done using combinations of CD₄⁺/CD₃⁺/CD₁₉⁺, CD₄₅⁺/CD₅₆⁺/CD₃⁺, CD₃⁺/CD₄⁺/CD₈⁺ antibodies (BDTM Biosciences; San Diego, CA, USA) through flow cytometer. 50 µl of whole blood was added to diluted (1X) FACS lysing solution (BDTM Biosciences; San Diego, CA, USA) in dark to lyse the red blood cells. 20 µl of Tri test reagent (CD₄⁺/CD₈⁺/CD₃⁺; CD₃⁺/CD₁₉⁺/CD₄₅⁺; CD₃⁺/CD₁₆₊₅₆⁺) was pipetted into the bottom of the BDTM TruCOUNT tube. 50 µl of well mixed anti-coagulated blood was added into the bottom of the tubes with the help of a pipette. The tubes were vortexed and incubated for 15 min in the dark at RT. Finally, 450 µl 1X FACS lysing solution was added to each tube, incubated for 15 min. in dark at RT and subjected for analysis on the flow cytometer.

Analysis of Th1/Th2 cytokines: Two ml of heparinized blood

was diluted in 9 volumes of RPMI 1640 medium (Invitrogen Co., Carlsbad, CA, USA) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. Diluted blood was incubated at 37°C for 3 days in tissue culture plates (1 ml/well) (Nalgene-Nunc Inc., Roskilde, Denmark) containing 10 mg/ml purified protein derivative (PPD, Span Diagnostics Ltd, Surat, India) in duplicates (15). Culture supernatants were collected for determination of Th1/Th2 cytokines was performed by Multiplex Cytometric Bead Array analysis kit following all the necessary instructions from the manufacturer. Data acquisition and analysis were carried out on a flow cytometric platform using BD CBA software (San Diego, CA, USA).

Statistical Analysis: Student's 't'-test using SPSS software package (SPSS Inc., Chicago, IL, USA) was employed for statistical analysis and a P value of ≤ 0.05 was considered to be significant.

RESULTS

Quantitative estimation of MTB DNA: A positive fluorescence signal in 5 out of 10 FFPE, 13 out of 40 tissue biopsies (Table 2) confirming the presence of MTBC DNA in the group C patients was observed with overall 36% positivity. However the utility of real time PCR assay in the known culture and ZN stain positive specimens (group B) showed 99% specificity in MTBC diagnosis as all the 27 from 28 samples reported in the study of this group were positive through LC PCR. The quantitative bacterial load ranged from 1.62 x 10⁻⁶ to 5.46 x 10² copies/µl (Table 3). The results observed in the group A (non MTBC) showed the specificity of LC PCR to be 100 % as no positive signal was observed in the total studied 24 cases. The internal control of the real-time PCR assay was positive for all specimens, negating out the existence of possible inhibitions. With a positive PCR test there is a 98.95% probability of having tuberculosis (positive predictive value) with a minimum bacterial load of 1.6 copies/µl, and a negative PCR test gives a 95.19% negative predictive value i.e., chance of truly ruling out a MTBC infection in the GI tract. All the real

Table 1: Comparison of results of AFB culture / ZN staining and LC PCR of group A and B*

Sample type	Total no. of samples	AFB culture/ ZN staining		LC PCR	
		-ve	+ ve	-ve	+ ve
Controls (group A)	24	24	-	24	-
Patients (group A)	28	-	28	1	27

*AFB indicates acid fast bacilli; ZN, Ziehl Neelson; TB, tuberculosis and LC PCR, light cycler polymerase chain reaction

Table 2: Showing comparison of results of AFB Culture / ZN staining and LC PCR of group C*

Sample type	Site of involvement	Total no. of samples	AFB Culture/ ZN staining		LC PCR	
			- ve	+ ve	- ve	+ ve
*FFPE		10	10	-	05	05
	Abdomen	05	05	-	04	01
	Rectum	01	01	-	-	01
	Liver	02	02	-	01	01
	Ileum	02	02	-	-	02
Tissue Biopsy		40	40	-	27	13
	Cecum	10	10	-	05	05
	Colon	18	18	-	17	01
	Ileum	07	07	-	03	04
	Rectum	05	05	-	02	03

*FFPE Formalin fixed paraffin embedded tissue; ZN indicates Ziehl Neelson; TB, tuberculosis and LC PCR, Light Cycler Polymerase chain reaction

time PCR positive samples responded to the standard ATT - (HERZ)₂(HR)₁₀ therapy. ATT-(HERZ)₂(HR)₁₀ therapy is the standard anti-tuberculosis therapy comprising isoniazid (H) 5 mg/kg/day up to a maximum of 300 mg/day PO for 12 months;

rifampicin (R) 10 mg/kg/day up to a maximum of 600 mg/day PO for 12 months; ethambutol (E) 15 mg/kg/day up to a maximum of 1200 mg/day PO for initial 2 months; and pyrazinamide (Z) 25 mg/kg/day up to a maximum of 2000 mg/day PO for initial 2 months [(HERZ)₂(HR)₁₀].

Table 3: Depicting the total bacterial load in LC PCR positive samples*

S.NO	Sample Type	Site of involvement	Bacterial DNA load (copies/ μ l)
1	FFPE	Ileum	1.46×10^0
2	FFPE	Abdomen	4.83×10^0
3	FFPE	Ileum	1.65×10^1
4	FFPE	Rectum	1.62×10^{-6}
5	Tissue biopsy	Rectum	1.60×10^0
6	Tissue biopsy	Ileum	5.44×10^{-1}
7	Tissue biopsy	Ileum	7.05×10^{-1}
8	Tissue biopsy	Cecum	6.07×10^0
9	Tissue biopsy	Ileum	5.46×10^2
10	Tissue biopsy	Ileum	5.82×10^0
11	Tissue biopsy	Cecum	4.23×10^0
12	Tissue biopsy	Cecum	2.51×10^{-1}
13	Tissue biopsy	Cecum	6.20×10^{-1}
14	Tissue biopsy	Colon	3.32×10^0
15	Tissue biopsy	Rectum	2.48×10^0
16	FFPE	Liver	3.98×10^0
17	Tissue biopsy	Cecum	1.25×10^{-2}
18	Tissue biopsy	Rectum	3.92×10^{-1}

*FFPE Formalin fixed paraffin embedded tissue and LC PCR, Light Cycler Polymerase chain reaction

Lymphocyte subset enumeration studies

(Immunophenotyping): Lymphocyte subset enumeration studies (Immunophenotyping) revealed depletion in the total CD₄⁺ absolute counts i.e. 437.36 of GITB positive group C patients as compared to controls i.e. 991.47, however, a slight decrease in the CD₈⁺ absolute count (738.44) of GITB positive group C patients was also reported in comparison to the age and gender matched control group values (950.71). However, absolute counts of CD₄₅⁺, CD₃⁺, CD₁₉⁺, CD₁₆₊₅₆⁺ in the GITB

Fig 1: demonstrating the comparative data of lymphocyte subset enumeration studies of the group C culture and AFB negative samples positive for MTBC infections through LC PCR 2.0 with age and gender matched controls. Data is represented in the form of absolute counts. GITB represents gastrointestinal tuberculosis.*P=0.05

positive group C patients did not show significant difference from their respective control values (Fig 1).

Multiplex cytometric bead array analysis for human Th1/Th2 cytokines: A significant increase in the levels of IFN- γ and TNF- α cytokines in the culture supernatant of GITB positive group C patients was observed in comparison to the control levels. The mean levels of IL-2, IFN- γ and TNF- α cytokines observed were 654.84 pg/ml, 1043.78 pg/ml, 833.78 pg/ml while for IL-4, IL-6, and IL-10 were 536.37 pg/ml 463.21 pg/ml 536.85 pg/ml respectively (Fig. 2).

Fig 2: showing the cytokine profile (IL-2, IFN- γ , TNF- α , IL-4, IL-6, IL-10) of the LC PCR 2.0 positive samples for MTBC infections. Study was performed culture supernatant of lymphocytes treated with PPD (10 mg/ml) for 3 days. Data is represented in the form of concentration (pg/ml) and compared with age and gender matched controls. GITB represents gastrointestinal tuberculosis. * P=0.05

DISCUSSION

GITB is often underrated and yet, any kind of delay in prompt initiation, may lead to treatment failure or to developing antibiotic resistance. The significant morbidity and mortality observed in GITB is due to its imprecise features which do not readily suggest a specific diagnosis (3). This abdominal form of TB has an insidious course like any other chronic infectious disease without any specific laboratory, radiological or clinical findings. Due to this non-specificity and great difficulties in its diagnosis a number of rapid investigative methods have been surfacing out to aid in the diagnosis of GITB employing a diverse MTB genomic targets including the IS 6110 insertion sequences (16-19). Undeniably, the PCR systems developed so far have shown good levels of sensitivity (90 to 100%) only on AFB smear -positive samples (20). Our investigation exhibit that real time detection technology using FRET probes present superior sensitivity over conventional detection methodologies for rapid diagnosis of GITB. The results obtained for the group A & B depicted the accuracy of the real time assay as demonstrated by the congruency in the

both qualitative and quantitative results (Table 1). One negative result obtained in group B may be due to the uneven distribution of the MTBC in the tissue. The overall positive index of the samples of group C tested was found to be 36% (Table 2, 3).

Human infection with MTBC displays a spectrum of clinical manifestations that reflect the efficacy of the immune response. Antigen-specific CD4⁺ T lymphocytes are known to be the main effector cells against these infections due to their ability to produce cytokines that activate macrophages and maintain optimal CD8⁺ T cell responses (21, 22). Apart from providing immunologic resistance to MTBC, these cells are also involved in disease immuno-pathology (14, 23), due to which there is an inverse correlation between the impairment in the specific lytic activity of cells from TB patients and the severity of the disease (24). Moreover, differences in cytokine expression in particular, balance between Th1 and Th2 cytokine is likely to determine whether the infection progresses, resolves, or becomes latent (11). *Mycobacterial* infections were also shown to be associated with depletion in CD4⁺ T cells absolute count along with increased levels of circulating IFN- γ and TNF- α (21, 25-27). Studies have also reported that these increased IFN- γ and TNF- α levels may serve as a diagnostic marker for TB (28, 29). Consistent with these findings, we observed a depleted number of CD4⁺ cells in the LC PCR positive samples along with a significant increase in the levels of IFN- and TNF- α in culture supernatant in comparison to their respective control values confirming an opportunistic *Mycobacterial* infection (Fig 1, 2).

Our results signify the importance of molecular assays for better disease management and inconsequently helping the patient to aware of the infection without any delay. However, to establish the superiority of this novel technique for the MTBC diagnosis in various infectious states, it will be necessary to accumulate data from larger number of fractions with suspected tuberculosis infections. Above all advances, an integrated approach for the early identification of disease are the need of the hour. Given the critical balance between immune-mediated suppression of *Mycobacteria* and immune-mediated tissue pathology in TB, further in-depth understanding of these interactions would allow the development of increasingly specific immune-based interventions for prevention and treatment of TB.

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